Formation of the early canine CL and the role of prostaglandin E2 (PGE2) in regulation of its function: An in vivo approach

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Abstract: The mechanisms governing corpus luteum (CL) function in domestic dogs remain not fully elucidated. The upregulated expression of cyclooxygenase 2 and prostaglandin (PG) E2 synthase (PGES) at the beginning of the canine luteal phase indicated their luteotrophic roles, and the steroidogenic activity of PGE2 in the early canine CL has been confirmed in vitro. Recently, by applying a cyclooxygenase 2 (COX2)-specific inhibitor (firocoxib [Previcox]; Merial) from the day of ovulation until the midluteal phase, the luteotrophic effects of PGs have been shown in vivo. This is a follow-up study investigating the underlying endocrine mechanisms associated with the firocoxib-mediated effects on the canine CL. Experimental groups were formed with ovariohysterectomies performed on Days 5, 10, 20, or 30 of firocoxib treatments (10 mg/kg bw/24h; TGs = treated groups). Untreated dogs served as controls. A decrease of steroidogenic acute regulatory (STAR) protein expression was observed in TGs. The expression of PGE2 synthase was significantly suppressed in TGs 5 and 10, and both PGE2 and PGF2 levels were decreased in luteal homogenates, particularly from CL in TG 5. Similarly, expression of the prolactin receptor (PRLR) was diminished in TGs 5 and 20. The expression of PGE2 receptors PTGER2 (EP2) and PTGER4 (EP4), the PG- transporter (PGT) , and 15-hydroxy PG dehydrogenase (HPGD) was not affected in TGs. Our results substantiate a direct luteotrophic role of PGs in the early canine CL, i.e., by upregulating the steroidogenic machinery. Additionally, the possibility of an indirect effect on PRL function arises from the increased prolactin receptor expression in response to PGE2 treatment in canine lutein cells observed in vitro.

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Short title: Role of PGE2 in canine CL: In vivo study

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Abstract

The mechanisms governing corpus luteum (CL) function in domestic dogs remain not fully elucidated. The upregulated expression of cyclooxygenase 2 (COX2) and prostaglandin (PG) E2 synthase (PGES) at the beginning of the canine luteal phase indicated their luteotrophic roles and the steroidogenic activity of PGE2 in the early canine CL has been confirmed *in vitro*. Recently, by applying a COX2 specific inhibitor (firocoxib, Previcox®, Merial) from the day of ovulation until the mid-luteal phase, the luteotrophic effects of prostaglandins have been shown *in vivo*. This is a follow-up study investigating the underlying endocrine mechanisms associated with the firocoxib-mediated effects on the canine CL. Experimental groups were formed with ovario-hysterectomies performed at days 5, 10, 20 or 30 of firocoxib-treatments (10mg/kg bw/24h; TGs = treated groups). Untreated dogs served as controls. A decrease of STAR protein expression was observed in TGs. The expression of PGES was significantly suppressed in TGs 5 and 10, and both PGE2 and PGF2α levels were decreased in luteal homogenates, particularly from CLs in TG 5. Similarly, expression of the prolactin receptor (PRLR) was diminished in TGs 5 and 20. The expression of PGE2-receptors PTGER2 (EP2) and PTGER4 (EP4), the PG-transporter (PGT) and 15-PG-dehydrogenase (HPGD) was not affected in TGs. Our results substantiate a direct luteotrophic role of PGs in the early canine CL, *i.e.*, by upregulating the steroidogenic machinery. Additionally, the possibility of an indirect effect on PRL function arises from the increased PRLR expression in response to PGE2 treatment in canine lutein cells observed *in vitro*.

Keywords: Dog (Canis familiaris), prostaglandins, corpus luteum

Introduction

The mechanisms regulating luteal establishment and maintenance in the domestic dog (*Canis familiaris*) are still not well understood. Following the strong preovulatory luteinisation and ovulation, the canine corpus luteum (CL) develops from residual follicular cells. Cell populations known to contribute to luteal development in other species, *i.e.,* cells of the follicular wall-associated granulosa and the theca interna, likely contribute to the formation of the mature canine CL, even though there is no distinction between small and large lutein cells (reviewed in (13)). The central role of the CL in regulating canine reproduction is due to the fact that, in both pregnant and nonpregnant dogs, CLs are the only source of progesterone (P4) and are thus responsible for the establishment and maintenance of pregnancy. This is in contrast to other carnivores like felids, where progesterone of placental origin has been recently suggested as an additional source of progesterone (2,26). Specific to the dog is also the lack of a uterine luteolysin of endometrial origin (9,24), which in absence of pregnancy can result in a luteal life span exceeding that observed during pregnancy (9).

Whereas during the second half of the luteal phase the canine CL strongly depends on hypophyseal support, with PRL being the crucial luteotrophic hormone as early as day 25 after the preovulatory LH peak (22,23,25), early luteal development appears to be characterized by a period of transitional gonadotropic independence (12,13,23). During this time, locally produced (intra-CL) prostaglandins (PGs) appear to be amongst the most important luteotrophic factors. Thus, formation of the canine CL is associated with increased cyclooxygenase 2 (COX2, PTGS2) and PGE2-synthase (PGES, PTGES) expression (14,19,21), accompanying progressively increasing expression levels of the steroidogenic acute regulatory (STAR) protein and 3β-hydroxysteroid-dehydrogenase (3βHSD, HSD3B2) and dynamically rising circulating P4 levels. In line with these findings, we were able to show that the PTGES-derived PGE2 significantly upregulates P4 synthesis in canine luteal cells isolated from the early CL (15). This effect seems to be mediated at the level of STAR-

dependent steroid substrate provision, rather than at the level of enzymatic activity of 3βHSD and P450 side-chain cleavage enzyme (P450scc, CYP11A1), as concluded from their unaltered expression in response to treatment with PGE2. Similar effects were observed in other species, such as pig, sheep and human (3,7,29), in which PGE2 positively affected P4 production by acting through the cAMP/PKA pathway, which is the main cell signalling route involved in STAR-mediated steroidogenesis. The luteotrophic efficiency of PGE2 has been shown to be similar to that of LH (29).

Most recently, in experiments applying a selective COX2 (PTGS2) inhibitor (firocoxib, Previcox®, Merial) during the early canine dioestrus phase, compelling *in vivo* evidence has been provided for luteotrophic effects of PGs in the canine CL (11). In dogs treated for up to 30 days after ovulation with this compound, inhibition of the steroidogenic apparatus was achieved. This was clearly indicated by significantly reduced areas of luteal cell nuclei, decreased expression of 3βHSD, and lower mean P4 concentrations in treated dogs.

In order to gain additional information on the underlying endocrine mechanisms associated with the firocoxib-mediated effects on canine CL, the present experiments assessed the effects of firocoxib on the luteal expression of STAR, COX2 (PTGS2), PTGES and their respective PGE2 receptors, EP2 (PTGER2) and EP4 (PTGER4), 15-hydroxyprostaglandin dehydrogenase (HPGD, the enzyme responsible for enzymatic deactivation of PGs) and prostaglandin transporter (PGT, SLCO2A1). In parallel, the luteal content of PGE2 and PGF2α was measured. Furthermore, since known luteotrophic hormones could potentially modulate each other's functions, the expression of PRL-receptor (PRLR), LH-receptor (LHR) and the progesterone receptor (PGR) was investigated.

Additionally, taking into account the role of PRL as the main luteotrophic factor during the second half of canine dioestrus, and the recently presented evidence showing increasing expression of its receptor during the early luteal phase (18), the expression of PRLR was investigated by assessing mRNA obtained from primary luteal cells isolated during the early

78 luteal phase, which had been treated with PGE2 in previous experiments (15).

79

80 **Material and Methods**

81 **Tissue sampling**

82 Tissue materials were the same as those used in our previous study (11). All animal
83 experiments were approved by the responsible ethics committee of the University of Warmia
84 and Mazury in Olsztyn, Poland.

85 In total, thirty (n=30) bitches of various breeds aged 2-7 years were used for the present
86 study. They were randomly divided into four experimental groups with ovario-hysterectomy
87 performed on days 5 (n=4), 10 (n=3), 20 (n=3) and 30 (n=3) of the treatment and designated
88 as TGs (treatment groups 1-4, respectively). During the treatment periods, dogs received 10
89 mg/kg daily of firocoxib (a highly selective COX2 inhibitor marketed as Previcox® by Merial
90 Ltd.), which was double the recommended dose of 5 mg/kg body weight per day. Ovario-
91 hysterectomies were performed on the last day of treatment. Additionally, untreated dogs
92 received a placebo and served as controls (CGs = control groups). Five CGs were designated:
93 days 0 (n=3), 5 (n=5), 10 (n=3), 20 (n=3) and 30 (n=3) post ovulation (p.o.). Day 0 was the
94 day of ovulation, determined as the day when peripheral P4 reached levels >5 ng/ml, as
95 measured every two days using a commercially available chemiluminescence kit
96 (Progesteroene II®, Roche Diagnostics, Mannheim, Germany). Additionally, the oestrous
97 cycle stage was monitored by examination of vaginal smears. Blood samples were collected
98 every 2 days following ovulation during the entire duration of the experiments for continuous
99 P4 measurements and stored at -20^0 C until evaluation. The P4 concentrations are presented
100 elsewhere (11).

101 Following ovario-hysterectomy, CLs were trimmed of connective tissue and cut into small
102 pieces. Subsequently, one piece was preserved for RNA isolation by incubation for 24 h at
103 $+4^O$C in RNAlater® (Ambion Biotechnology GmbH, Wiesbaden, Germany) and then stored at

-80^{O}C until RNA isolation. Another piece of the tissue material was fixed for 24 h at +4^{O}C with 10% phosphate-buffered formalin and paraffin-embedded using a standard procedure.

Primary luteal cell cultures

The mRNA obtained from a previous study (15) was used in the present experiments to gain additional information on PRLR mRNA expression in canine lutein cells in response to PGE2-treatment. In that study, canine primary lutein cells were isolated from 15 healthy bitches submitted for routine ovario-hysterectomies, which were performed during the early luteal phase, 7-14 days after the clinical signs of heat had ceased. Briefly, 0.15% Collagenase (Sigma-Aldrich Chemie GmbH, Buch, CH) was applied to isolate cells from luteal tissues collected from each animal. A 75μm cell strainer (BD Biosciences, Basel, CH) was used to remove undissociated tissue fragments. After washing in culture medium (DMEM/F12, pH 7.2-7.4 with 10% heat-inactivated FBS, 100U/ml penicillin and 100μg/ml streptomycin, and 1% ITS; all from Chemie Brunschwig AG, Basel, CH), cells were suspended in culture medium and seeded directly into 6-well plates and cultured in a humidified incubator at 37^{o}C under 5% CO_2 in air. Importantly, the cells were not passaged (trypsinized) prior to the experiments: "passage 0" cells were used. Cells were treated in serum-free medium. The steroidogenic identity of the isolated cells had been confirmed by performing anti-3βHSD and anti-STAR immunoflorescence staining. When stimulated for 6 h with either 0.5 mM N6,2-dibutyryladenosine-3,5-cyclic monophosphate (dbcAMP; used for positive control) or 20 μM PGE2 (both Sigma-Aldrich Chemie GmbH, Buchs, Switzerland), cells exhibited upregulated expression of STAR mRNA and protein, as well as strongly increased P4 output.

Reverse Transcription (RT), real time (TaqMan) PCR and data evaluation

TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) was used to isolate total RNA according to the instructions of the manufacturer. The concentration of RNA was determined with a NanoDrop 2000C® spectrophotometer (Thermo Fisher Scientific AG, Reinach, Switzerland). In order to remove any potential genomic DNA contamination frequently occurring with the use of TRIzol®, prior to reverse transcription (RT), all samples were DNase-treated using RQ1 RNase-free DNase (Promega, Dübendorf, Switzerland), following the supplied protocol. For RT reaction, random hexamers were used as primers, which together with other reagents for cDNA synthesis, were purchased from Applied Biosystems (Foster City, CA, USA). Our previously published standard RT protocol was applied (21). All reactions were conducted in an Eppendorf Mastercycler® (Vaudaux-Eppendrf AG, Basel, Switzerland). The conditions applied were as follows: 8 min at 21°C, 15 min at 42°C, finally for 5 min at 99°C to stop the enzymatic reaction.

The luteal expression of target genes was semi-quantified by real time (TaqMan) PCR performed in an automated ABI PRISM 7500 Sequence Detection System (Applied Biosystems), with the previously published protocol and the manufacturer's instructions (17,21). For all samples, cDNA corresponding to 200 ng of total RNA per sample was used in duplicate in 96-well optical plates (Applied Biosystems). Fast Start Universal Probe Master (ROX®) (Roche Diagnostics AG, Switzerland) was used in reactions that were run under the following conditions: denaturation at 95 °C for 10 min, 40 cycles at 95°C for 15 sec and 60°C for 60 sec. Water instead of cDNA and the so-called RT minus control (*i.e.*, samples that were not reverse-transcribed) were run as negative controls.

Three reference genes, GAPDH, 18SrRNA and cyclophilin, were used in the comparative CT method (ΔΔCT method) according to the instructions of the ABI 7500 Fast Real-Time PCR System manufacturer and as described previously (17,21). By applying the CT slope method, PCR reactions were set up to achieve approximately 100% reaction efficiency. Selected PCR products were sent for commercial sequencing (Microsynth, Balgach, Switzerland). The

primers and 6-carboxyfluorescein (6-FAM)- and 6-carboxytetramethyl-rhodamine (TAMRA)-labelled TaqMan probes were designed using Primer Express Software (Version 2.0, Applied Biosystems) and purchased from Microsynth (Table 1). The canine-specific cyclophilin A TaqMan gene Expression Assay used was obtained commercially from Applied Biosystems (Prod. No. Cf03986523_gH).

Immunohistochemical expression of STAR and PRLR

The standard immunoperoxidase immunohistochemistry (IHC) protocol was applied according to our previously published procedure (16,18). Primary antisera were: rabbit polyclonal anti-STAR (a gift from Dr. DM Stocco, Texas Tech University Health Sciences Center, Lubbock, TX, USA; (4)) used at 1:3000 dilution, and polyclonal goat antigen affinity-purified IgG fraction against human PRLr (R&D Systems Europe Ltd), dilution 1:50. Serum from non-immunized rabbit or goat IgG irrelevant antibodies I-5000, from Vector Laboratories Inc., Burlingame, CA940101, CA, USA, served as negative/isotype controls, respectively. Depending on the primary antiserum, 10% horse serum or 10% normal goat serum was used for blocking of nonspecific binding sites. Secondary antibodies were: goat anti-rabbit IgG BA-1000 and horse anti-goat IgG BA-9500, both from Vector Laboratories Inc. (Burlingame, CA, USA), used at 1:100 dilution. Signals were enhanced with avidin-biotin-peroxidase complex (Vector Laboratories Inc.) and detected using a Liquid DAB+ substrate kit (Dako Schweiz AG, Baar, Switzerland). Sections were counterstained with hematoxylin and embedded in Histokit (Assistant, Osterode, Germany)

Luteal PGE2 and PGF2α extraction and determination

Extraction of luteal prostaglandins was done according to the previously described protocol (27). Briefly, luteal fragments weighing 30-50 mg were stored at -80°C. After thawing, each tissue sample was homogenized in a glass vial using a tissue disruptor with 400 µl of a Tris

buffered saline containing proteins and sodium azide as preservative, acidified by addition of 45 µl 1 N HCl. After adding 3 ml of ethyl ether to the samples, they were vortexed for 10 min and incubated at -20°C for 4 h. Afterwards, the supernatant was collected and evaporated to dryness under a stream of nitrogen at 40°C. Finally, 400 µl of the Tris buffered saline was added, mixed and allowed to sit for 15 min at 20°C. The samples were stored at -20°C until the immunoassay was run.

For $PGF_{2\alpha}$ and PGE_2 measurements, the commercial $PGF_{2\alpha}$ high sensitivity EIA kit and the PGE_2 high sensitivity EIA kit (both from ENZO Life Sciences Inc., Farmingdale, NY, USA) were used, and run according to the manufacturer's instructions.

The sensitivity of the PGF2α assay was 0.98 pg/ml. The cross-reactivity for various PGs and their metabolites was as follows: PGF2α 100%, PGF1α 11.82%, PGD2 3.62%, 6-keto-PGF1α 1.38%, PGI2 1.25% and PGE2 0.77%. The inter- and intra-assay variation coefficients were 10.8% and 8.6%, respectively. The sensitivity of the PGE2 assay was 8.26 pg/ml. The cross-reactivity for various prostaglandins and their metabolites was as follows: PGE2 100%, PGE1 70%, PGE3 16.3%, PGF1α 1.4%, PGF2α 0.7% and 6-keto-PGF1α 0.6%. The inter- and intra-assay variation coefficients were 12.2% and 6.9%, respectively.

Statistical evaluation

Due to the uneven distribution of the real time PCR data, the geometric means (Xg) and deviation factors (DF) were calculated for the analysis of target gene expression. The PGE2 and PGF2α data are presented as the mean ± SD. The Kruskal-Wallis Test (a nonparametric ANOVA) followed by Dunn's Multiple Comparisons Test was applied for testing the effects of time on mRNA levels in all control samples (CGs). The effects of time on concentrations of PGE2 and PGF2α were calculated using one-way analysis of variance (ANOVA) followed by Tukey-Kramer Multiple Comparisons Test applied for testing the effects of time on

hormone levels in all control samples (CGs). In order to test for the effects of treatments, an unpaired two-tailed Student's *t*-test was performed; numerical data are presented either as Xg ± DF or mean ± SD. For all tests, the statistical software program GraphPad 3.06 (GraphPad Software Inc., San Diego, CA, USA) was used. $P < 0.05$ was considered statistically significant.

Results

Histological assessment of (peri)ovulatory canine follicles

Histological analysis of haematoxylin-eosin stained tissue cross sections confirmed that ovulation was in progress at day 0 (day of ovulation, P4 > 5ng/ml). Both freshly ovulated and preovulatory follicles were found (Fig. 1). The latter ones revealed advanced preovulatory luteinisation characterized by strong follicular wall folding. Morphological changes were clearly visible within the theca interna layers, the cells of which changed shape from elongated to rounded. The blood vessels containing luteinizing theca layers were separated from the follicular granulosa cells by clearly distinguishable basement membranes. Following ovulation, further luteinisation of theca interna cells, as well as luteinisation of granulosa cells, and remnants of the basement membranes were observed.

Semi-quantitation of gene expression

The expression of target genes was detectable in all tissue material available for the study. A significant effect of time was observed for the mRNA expression of STAR ($P<0.04$), COX2 (PTGS2) ($P<0.04$), PTGES (PGES) ($P<0.03$), EP2 (PTGER2) ($P<0.01$), EP4 (PTGER4) ($P<0.01$), HPGD ($P<0.01$) and PRLR ($P<0.01$) in control samples (CG) from ovulation throughout early dioestrus (Fig. 2 and 3). The expression of PGT, LHR and PGR did not differ significantly over time ($P>0.05$) (Fig. 3). The expression of STAR and PGES increased significantly ($P<0.05$) following ovulation, reaching the highest levels at day 5 p.o.. Their

231 expression remained statistically unaltered thereafter (Fig. 2). Similar effects were observed
232 for luteal PRLR expression, which was significantly (P<0.05) upregulated in CGs 5 and 20
233 compared with the day of ovulation (Fig. 3). The expression of COX2 and of the two PGE2
234 receptors, EP2 and EP4, which was strongly elevated at the beginning of the luteal phase,
235 continued to decrease towards the mid-luteal phase and was significantly (P<0.05)
236 downregulated at day 20 and/or 30 after ovulation (Fig. 2). The opposite effect was noted for
237 HPGD with values continuously increasing from ovulation until the end of the observation
238 period in CG 30 (P<0.05) (Fig. 2).
239 Similar to what was observed for most of the CGs, there was a high individual variability in
240 expression of genes following treatment with Previcox®. The expression of STAR, PGES and
241 PRLR was negatively affected by this treatment (Fig. 2 and 3). This effect was significant for
242 STAR mRNA expression in TG 5 (P<0.03), TG 20 (P<0.04) and TG 30 (P<0.02). The PGES
243 mRNA abundance was significantly suppressed in TG 5 (P<0.03) and TG 10 (P<0.04) and
244 that of PRLR in TG 5 (P<0.01) and TG 20 (P<0.04).
245 As for STAR and PRLR, similar effects to those observed at the mRNA level were observed
246 for expression of their respective proteins. These were detectable in all
247 immunohistochemically examined samples; the evenly distributed signals were localized to
248 the cytoplasm of lutein cells and appeared to be weaker when COX2 function, and thereby the
249 supply of prostaglandins, was compromised in Previcox®-treated dogs (Fig. 4 and 5).
250 The expression of other members of the prostaglandin family, *i.e.*, COX2, EP2, EP4, HPGD
251 and PGT, as well as of LHR and PGR, remained unaffected by the treatment (P>0.05) (Fig. 2
252 and 3).
253 The expression of PRLR was significantly upregulated in PGE2-treated lutein cells *in vitro*
254 (P<0.05) (Fig. 3F).
255
256 ***Luetal PGE2 and PGF2α content***

There was a significant ($P<0.02$) time effect observed for the luteal content of both PGE2 and PGF2α throughout the course of the experiment (Fig. 6). Their concentrations were strongly elevated in the CLs forming at ovulation with 394.9 ± 152.9 pg/mg tissue and 505.4 ± 94.5 pg/mg tissue for PGE2 and PGF2α, respectively. Whereas the PGF2α content was reduced after ovulation to 5.7 ± 1.7 pg/mg tissue, PGE2 remained high in early developing CLs at day 5 p.o. with 354.6 ± 140 pg/mg tissue. As presented in Fig. 6, the PGE2 concentration determined in luteal homogenates was significantly reduced in TG 5 ($P<0.04$) and TG 10 ($P<0.05$) of Previcox®-treated dogs. Similarly, the PGF2α content was further decreased in TG 5 ($P<0.03$).

Discussion

Morphologically and ultrastructurally, only one type of steroidogenic cell, displaying a high content of both endoplasmic reticulum and mitochondria (8), is present in the canine CL. Consequently, its cellular origin and the extent to which either of the cell types in the original population (*i.e.*, theca interna and granulosa) contribute to luteal formation have been subjects of continuous scientific debate (*e.g.*, (5,13)). Our histological analysis of canine ovarian tissues during ovulation revealed the presence of strongly luteinized preovulatory follicles as well as freshly-ovulated follicles. The post-ovulatory development was characterized by intense ingrowth of luteinizing theca interna cells accompanied by blood vessels. Within the inner part of the former follicle (*i.e.*, the *antrum folliculare*), luteinizing granulosa cells were observed, still separated from theca cells by remnants of basement membrane and not yet associated with blood vessels. Thus, this observation on the presence of luteinizing granulosa cells in the early canine CL provides a new insight into canine luteal physiology, even though the process of development of the uniform luteal cell population still remains to be elucidated.

Unequivocally, prostaglandins are among the most important and versatile factors regulating CL function in mammals. In dogs, the role of intraluteal PGs seems to be strongly linked to luteal formation rather than to luteolysis (14,21). This idea has been further strengthened by results obtained in the present study. Hence, expression of the so-called PG family members resembled their previously reported expression patterns (6,14,19), with early luteal development being characterized by increased expression of COX2 (PTGS2), PGES, PTGER2, PTGER4 and PGT, and gradually increasing HPGD expression towards mid-dioestrus. As a consequence thereof, increased availability of luteotrophic PGE2 is observed locally, *i.e.*, within the CL, as shown in the present study. This, in turn, leads to increased STAR protein expression and steroidogenic output.

The PGFS/AKR1C3, which is the only-known canine-specific isoform of PGF2α synthase, responsible for the direct conversion of PGH2 to PGF2α (6), is either absent or expressed at a very low level in the canine CL throughout dioestrus (14,20). This fact, in line with results obtained in the present study, is reflected in the very low PGF2α production within the CL, which is not sufficient to affect normal luteal function, even though PGF2α is luteolytic in dogs.

Whereas development of the CL was associated with low PGF2α but increased PGE2 content, the concentration of both PGs was high during ovulation, implicating their involvement in this process, and resembling the situation described in other species (10,28).

In addition to the recently described impairment of steroidogenic machinery in dogs treated with firocoxib during early dioestrus (11), in the present study, potential regulatory mechanisms involved in this process were investigated. Blocking COX2 decreased PGES expression and consequently reduced the PGE2 content in the CL, thereby impairing STAR mRNA and protein expression. This further indicates causality between the expression and functioning of these entities. It is noteworthy that functional coupling between COX2 and

306 PGES expression for PGE2 production, even though still not fully elucidated, was previously
307 observed in the brains of COX2-deficient mice (1). Interestingly, the expression and,
308 presumably, the function of the respective PGE2 receptors, PTGER2 and PTGER4, as well as
309 of PGT and HPGD, remained unaffected.
310 Making use of the availability of the partial canine-specific PRLR sequence cloned in our
311 laboratory (18), corresponding to the homologous portions of the extra- and trans-membrane
312 domains conserved in PRLR isoforms of other species, the impact of firocoxib on its
313 expression was investigated. Inhibition of COX2 significantly suppressed PRLR expression,
314 both at the mRNA and protein levels. Considering the function of PRL as the main
315 luteotrophic factor in the mature canine CL (22) and the possible role of its receptor as an
316 upstream regulator of STAR and/or 3βHSD expression (18), this is an interesting finding
317 since decreased expression of the PRLR might result in reduced sensitivity of the CL to
318 circulating PRL. The possible functional interrelationship between PGE2 and PRLR
319 expression is also surmised from the increased expression of PRLR-mRNA in PGE2-treated
320 canine luteal cells isolated from early CLs. However, before any final conclusion can be
321 drawn, the complete cDNA of canine PRLR and its potential isoforms need to be elucidated.
322 The high individual variability noted for the expression of target genes, especially in response
323 to treatment with Previcox®, may be indicative of compensatory mechanisms existing within
324 the canine CL and thus merits further investigations, possibly involving larger groups of
325 experimental animals.
326
327 Following up on our recent *in vivo* studies providing functional evidence for the luteotrophic
328 role of prostaglandins within the early canine CL, in this study the first insight is presented
329 into the underlying endocrine and molecular regulatory mechanisms. The previously
330 postulated (14,15,19) causality between the COX2 and the PGES-dependent synthesis of
331 PGE2 regulating STAR protein expression and function has now been clearly shown.

Thereby, the direct role of PGE2 in regulating early canine CL function, as characterized by decreased sensitivity towards gonadotrophic stimuli, has been further substantiated. Moreover, as indicated by *in vivo* and *in vitro* experiments (using canine lutein cell cultures), PGE2 seems to be involved indirectly in the PRL-mediated maintenance of canine CL function by potentiating PRLR expression. The latter pathway is a new regulatory mechanism not previously described for species, such as cattle and pigs, in which LH, and to a lesser extent PRL, is essential for luteal maintenance.

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Conflict of interests

Authors declare that there is no conflict of interest.

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Figure 1

Haematoxylin-eosin (HE) staining of canine (peri)ovulatory follicles, before ovulation (shown at lower and higher magnification) and after ovulation. Open arrowheads = blood vessels, solid arrowheads = basement membrane, open arrows = granulosa cells before ovulation and during luteinization (after ovulation), solid arrows = luteinizing theca interna cells, red arrowhead = cumulus-oocyte complex.

Figure 2

Expression of STAR, COX2, PGES, EP2 (PTGER2), EP4 (PTGER4) and HPGD as determined by semi-quantitative Real Time (TaqMan) PCR in luteal tissue samples of control (cont.) and Previcox® (Prev.)-treated dogs (Xg x $DF^{\pm 1}$). Kruskal-Wallis Test followed by Dunn's Multiple Comparison Test was applied to test the effects of time on gene expression in all control samples (dashed lines indicate $P<0.05$). Student's *t*-test was applied to test the effect of treatment on gene expression: (*) indicates $P<0.02$, (**) indicates $P<0.03$ and (***) indicates $P<0.04$.

Figure 3

Expression of PGT, LHR, PGR and PRLR as determined by semi-quantitative Real Time (TaqMan) PCR in (A-D) luteal tissue samples of control (cont.) and Previcox® (Prev.)-treated dogs (Xg x $DF^{\pm 1}$) and (E) PRLR expression in canine primary luteal cells collected from non-pregnant dogs during the early luteal phase and treated with PGE2. Untreated cells served as negative controls. Cell culture experiments were performed independently at least three times using cells isolated from different animals. The Kruskal-Wallis Test followed by Dunn's Multiple Comparison Test was applied to test the effects of time on gene expression in all control samples (dashed lines in "D" indicate $P<0.05$). Student's *t*-test was applied to test the

effect of treatment on gene expression. Bars with (*) differ at P<0.01, bars with (**) differ with P<0.04 and bars with (***) differ at P<0.05.

Figure 4

Immunohistochemical localisation of STAR protein in canine luteal samples in control and Previcox®-treated dogs. Representative pictures are shown for days 5, 20 and 30 after ovulation (p.o.). Positive staining is localized in cytoplasm of lutein cells.

Figure 5

Immunohistochemical localisation of PRLR in canine luteal samples in control and Previcox®-treated dogs. Representative pictures are shown for days 5 and 20 after ovulation (p.o.). Positive staining is localized in cytoplasm of lutein cells.

Figure 6

Concentrations of PGE2 and PGF2α in luteal homogenates of control (cont.) and Previcox® (Prev.)-treated dogs measured by EIA assay (pg/mg tissue; mean ± SD). One-way ANOVA followed by Tukey-Kramer Multiple Comparison Test was applied to test the effects of time on hormone concentrations in all control samples (dashed lines indicate P<0.05). Student's *t*-test was applied to test the effect of treatment on hormone concentration: (*) indicates P<0.03, (**) indicates P<0.04 and (***) indicates P<0.05.

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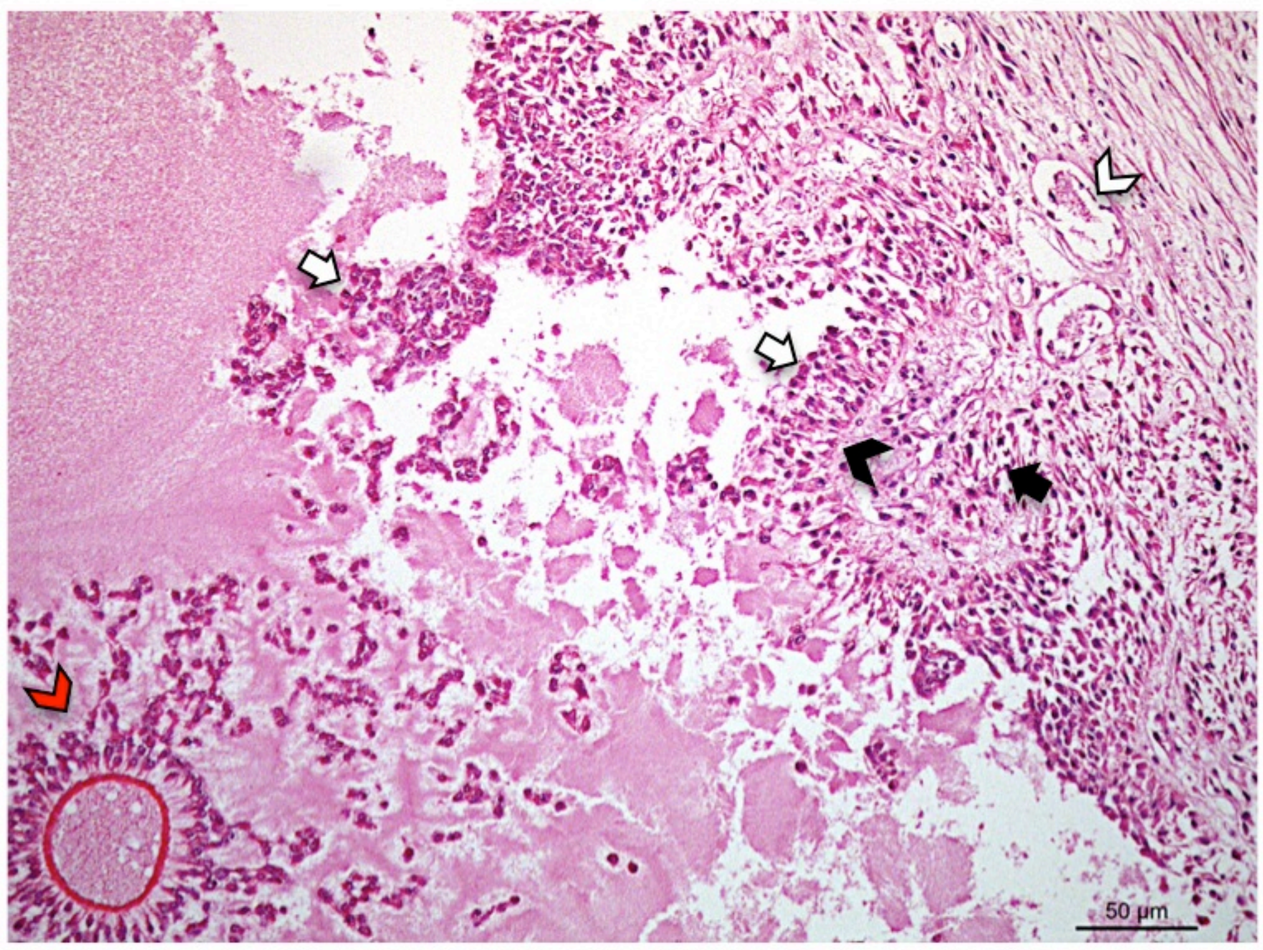
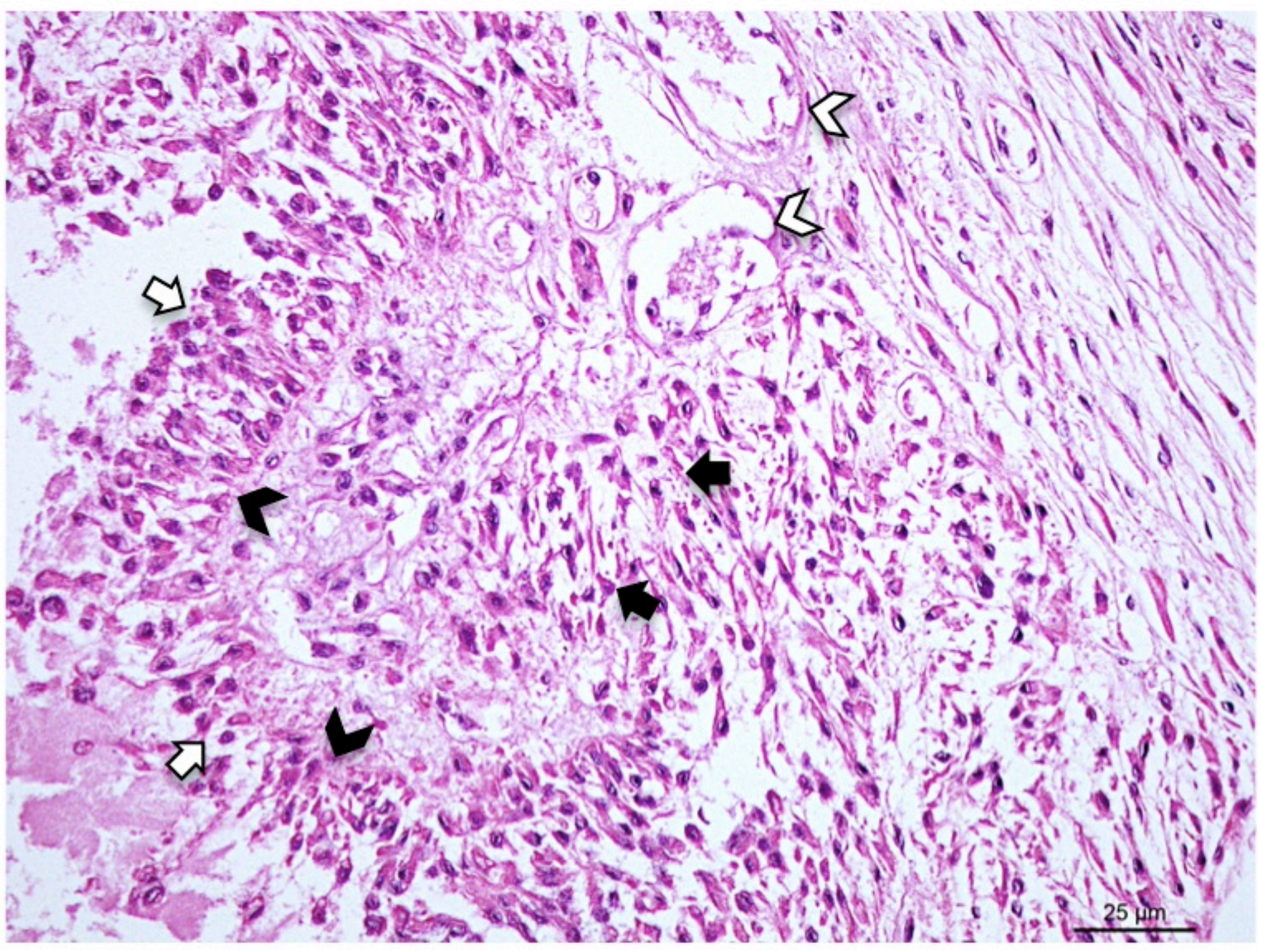
Before ovulation





After ovulation

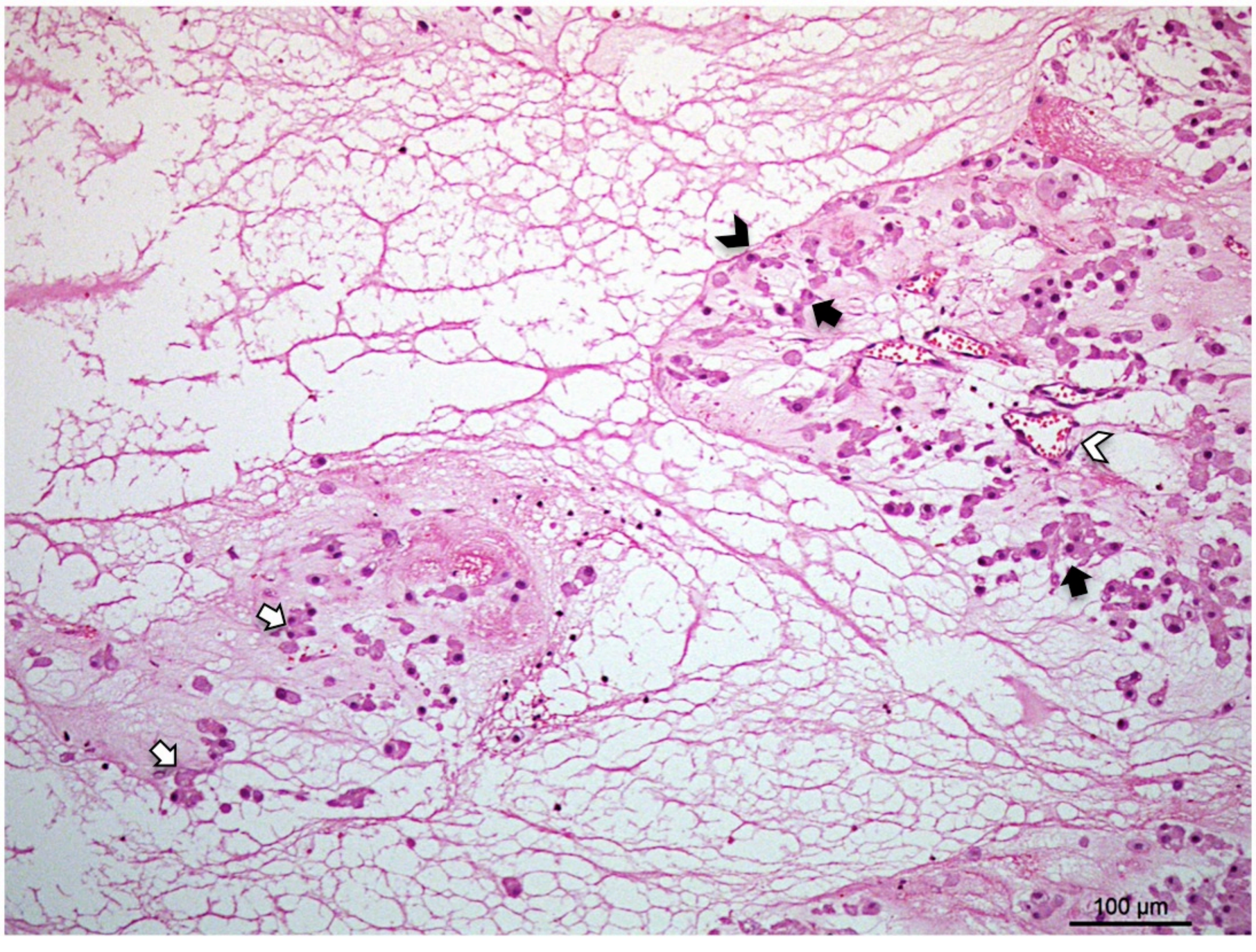


Figure 1

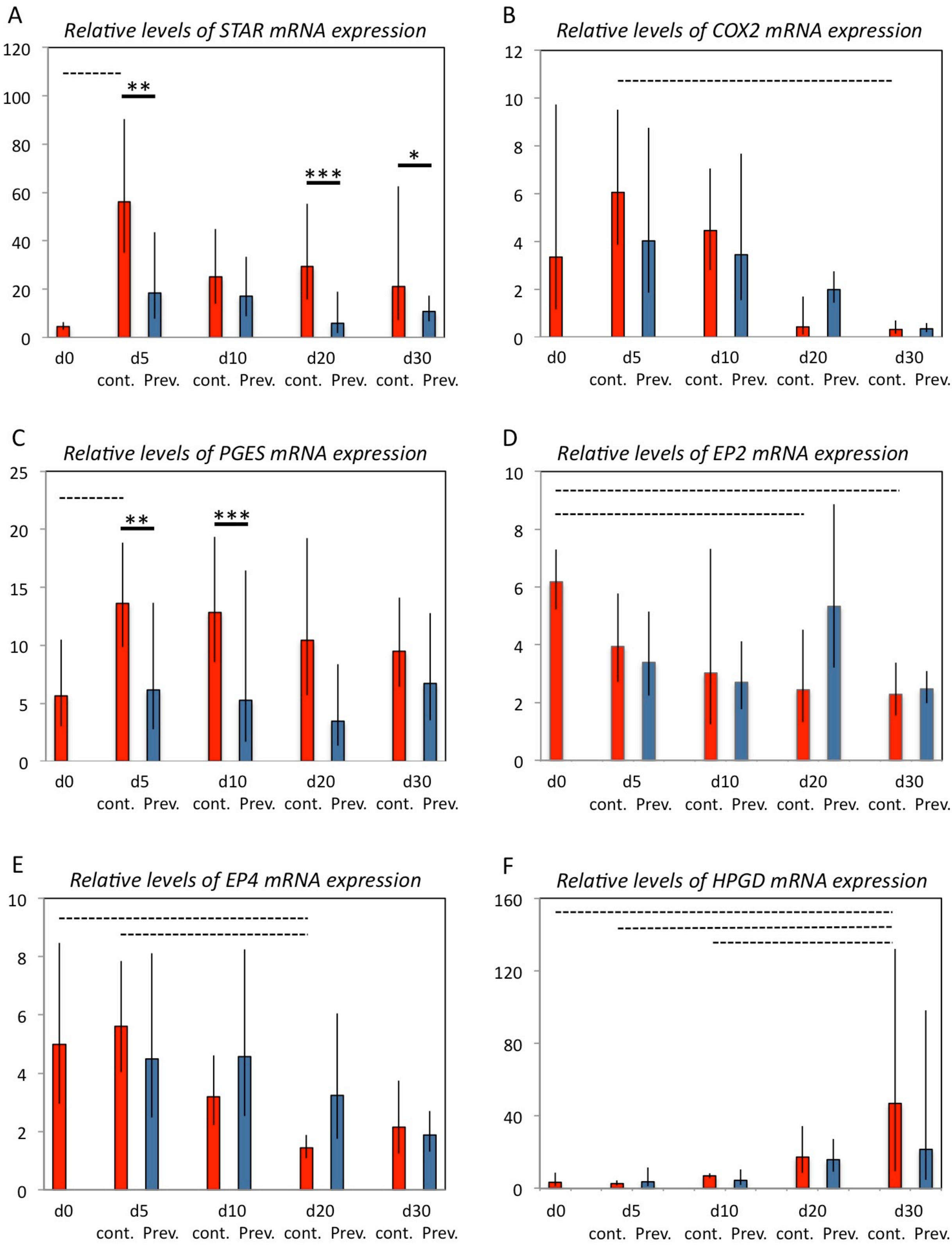

A
Relative levels of STAR mRNA expression
120
100
80
60
40
20
0
**
***
*
d0
d5
d10
d20
d30
cont. Prev. cont. Prev. cont. Prev. cont. Prev.
B
Relative levels of COX2 mRNA expression
12
10
8
6
4
2
0
d0
d5
d10
d20
d30
cont. Prev. cont. Prev. cont. Prev. cont. Prev.
C
Relative levels of PGES mRNA expression
25
20
15
10
5
0
**
***
d0
d5
d10
d20
d30
cont. Prev. cont. Prev. cont. Prev. cont. Prev.
D
Relative levels of EP2 mRNA expression
10
8
6
4
2
0
d0
d5
d10
d20
d30
cont. Prev. cont. Prev. cont. Prev. cont. Prev.
E
Relative levels of EP4 mRNA expression
10
8
6
4
2
0
d0
d5
d10
d20
d30
cont. Prev. cont. Prev. cont. Prev. cont. Prev.
F
Relative levels of HPGD mRNA expression
160
120
80
40
0
d0
d5
d10
d20
d30
cont. Prev. cont. Prev. cont. Prev. cont. Prev.

Figure 2

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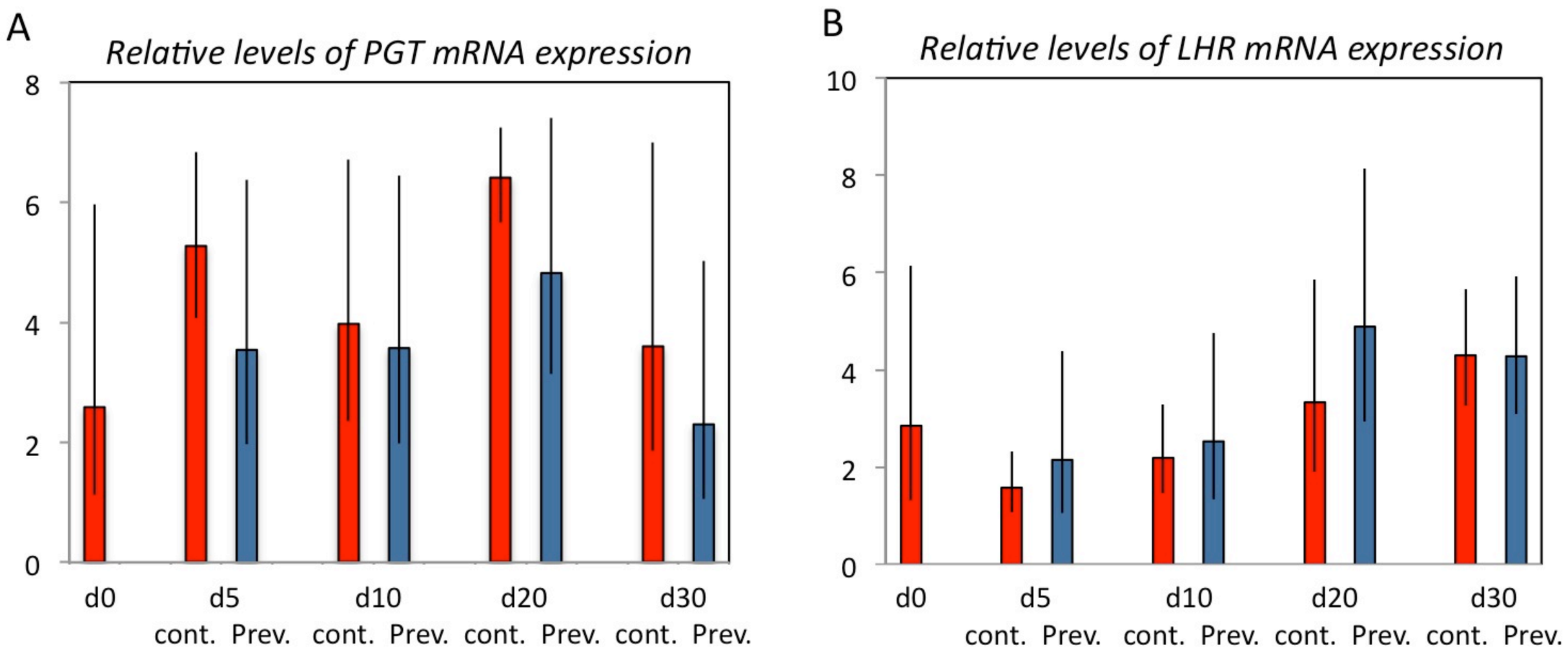

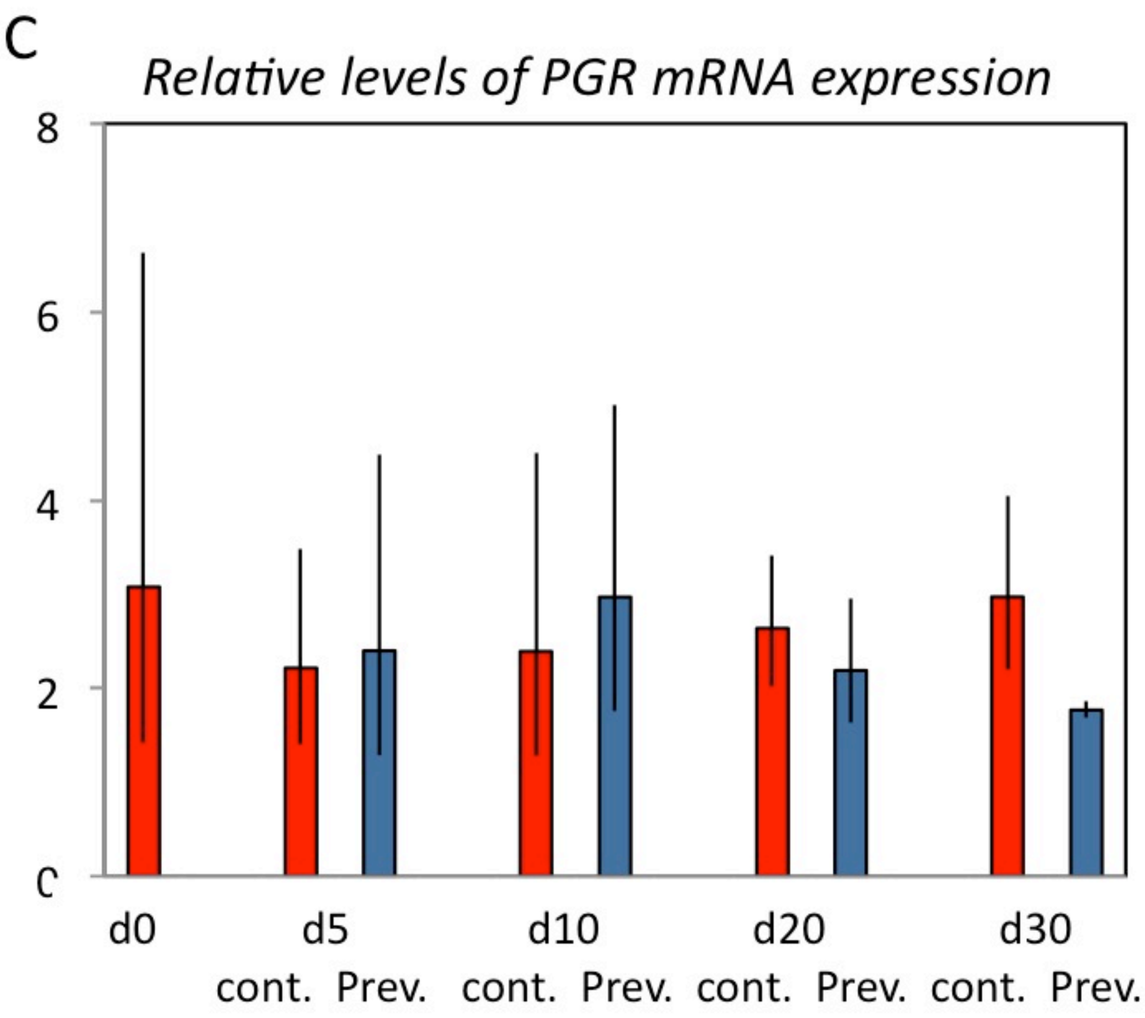

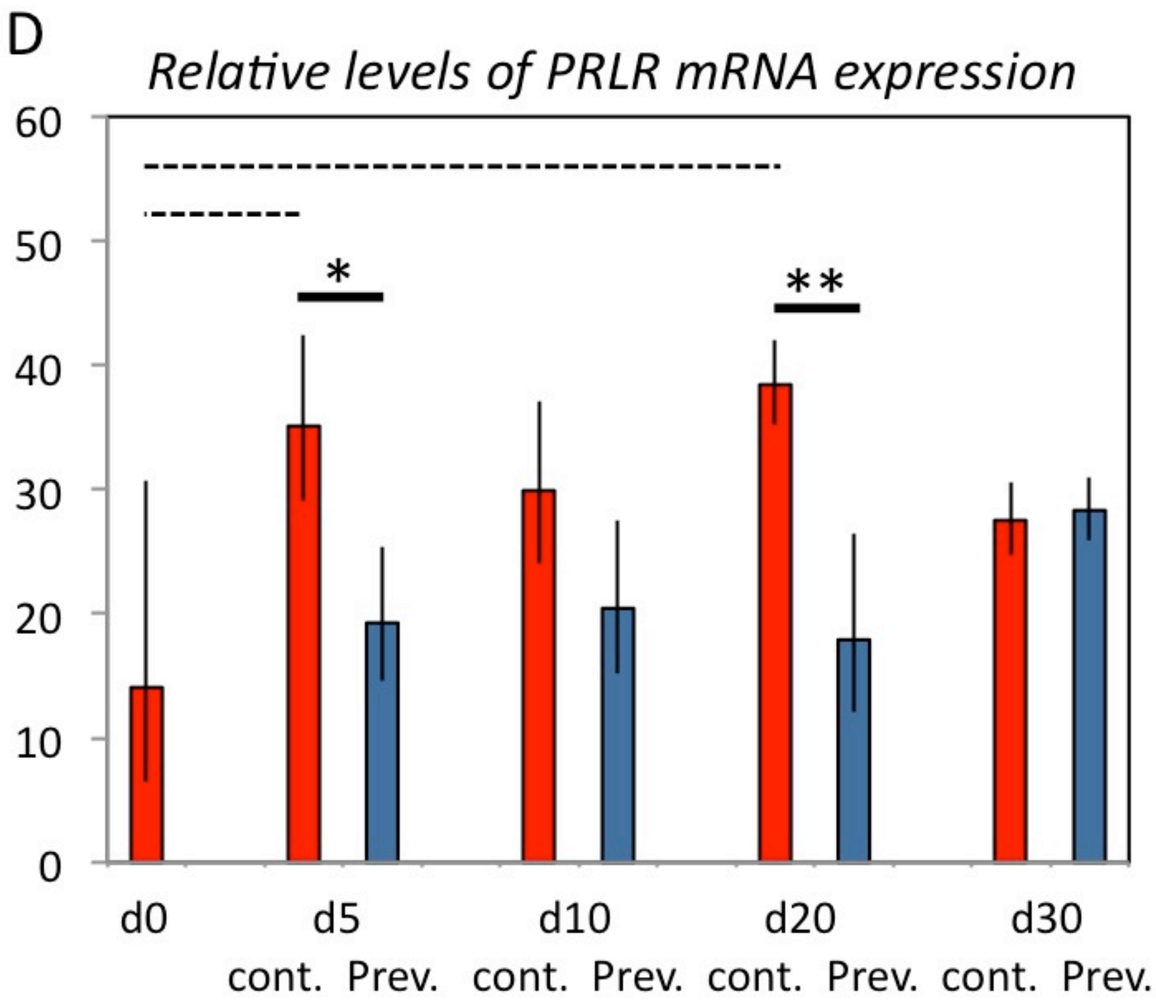

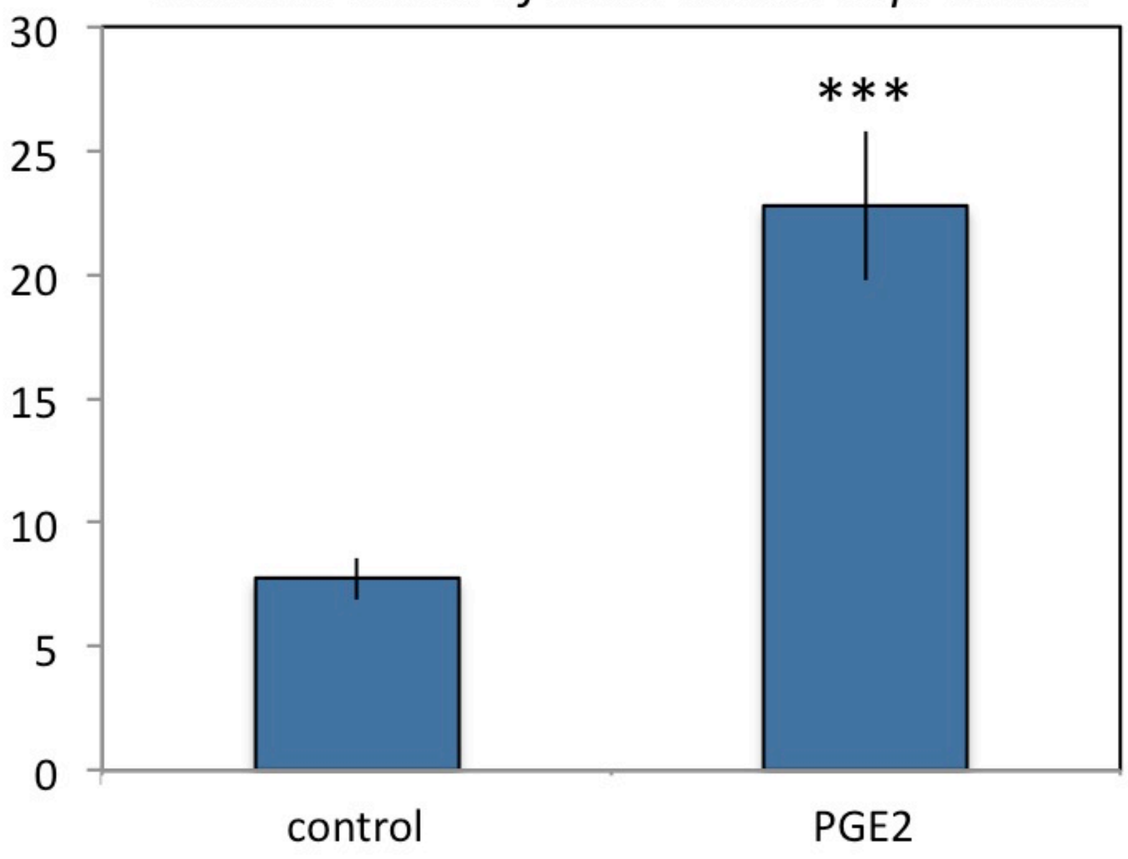

Figure 3

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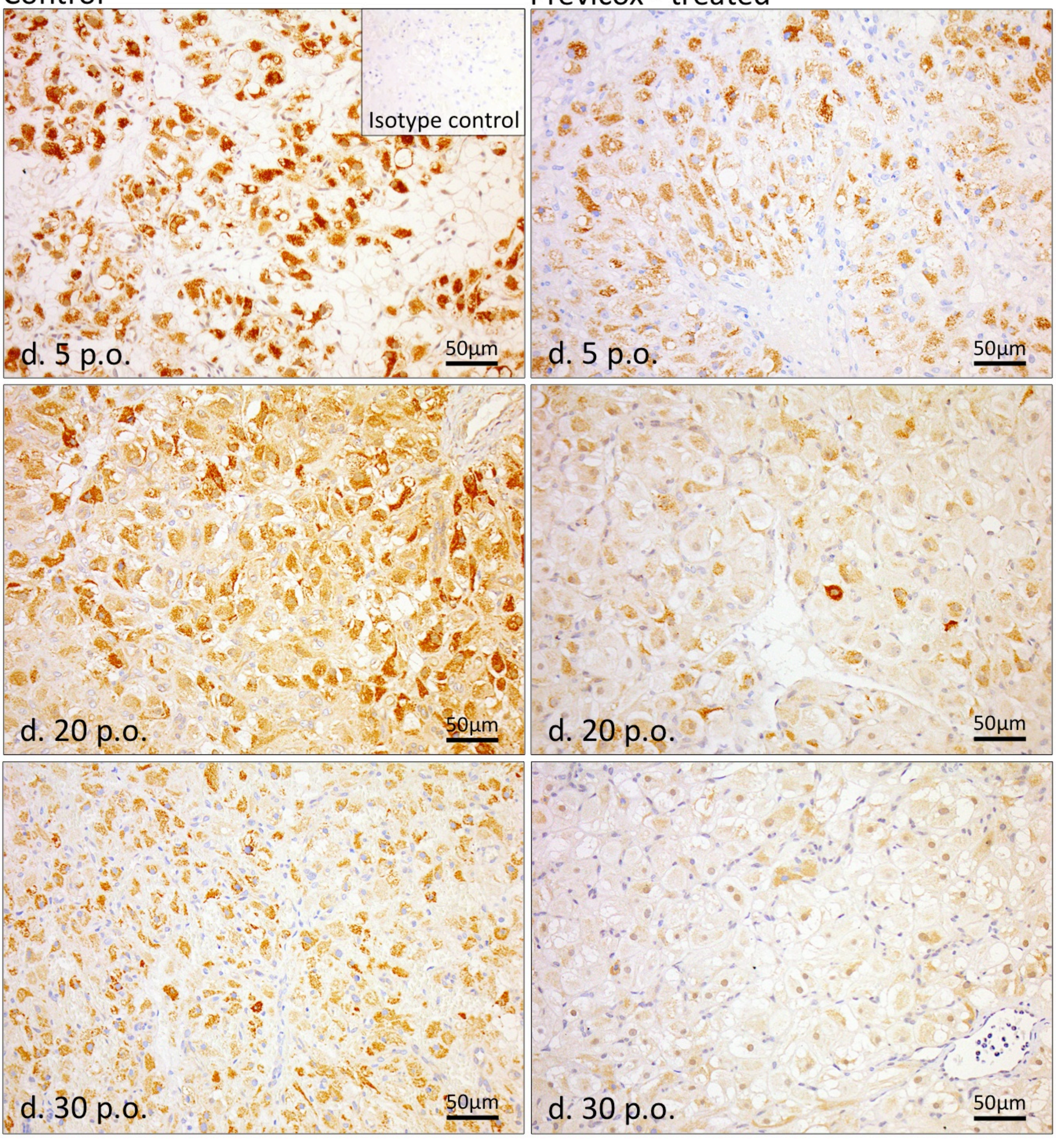

Figure 4

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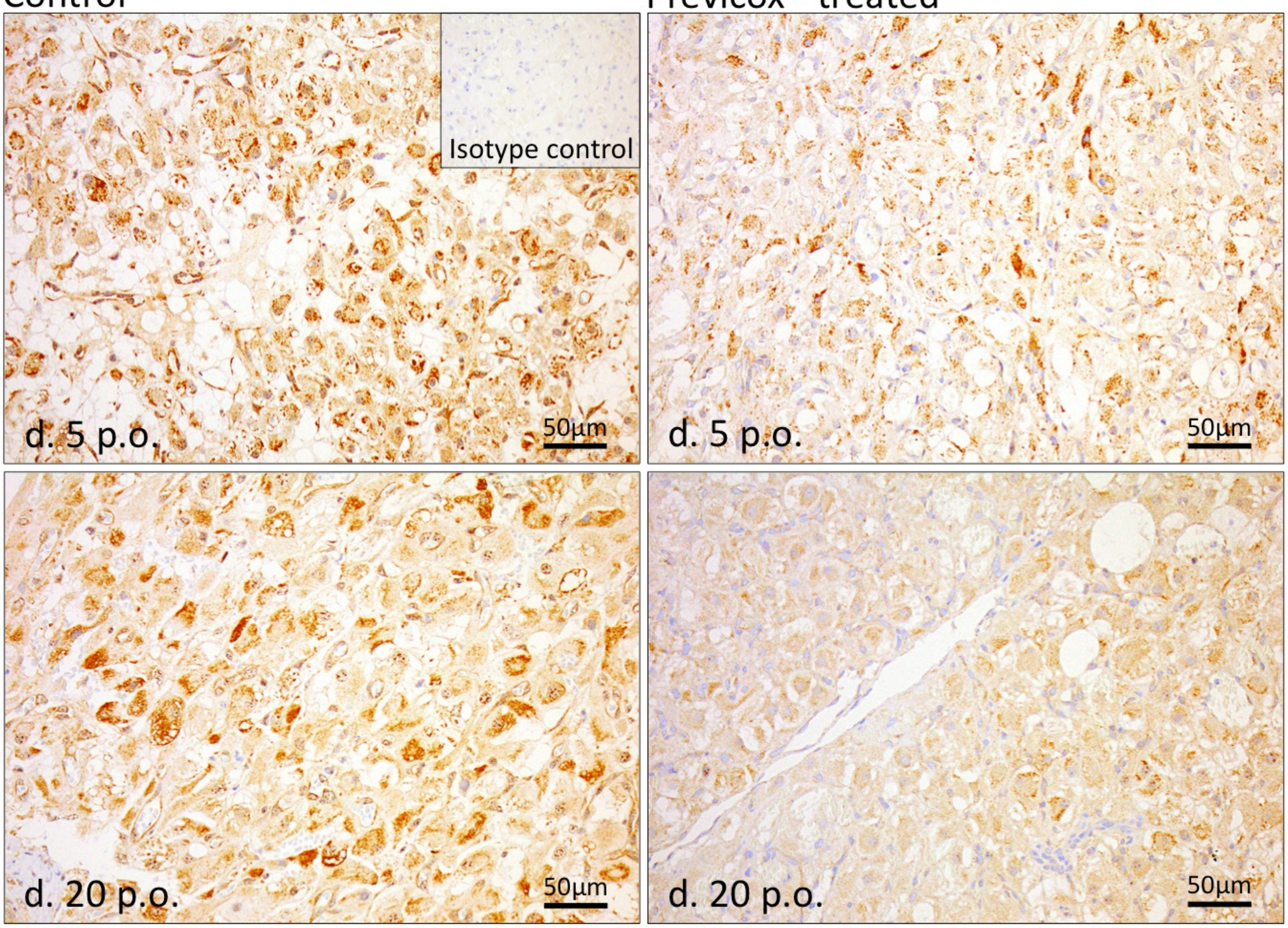
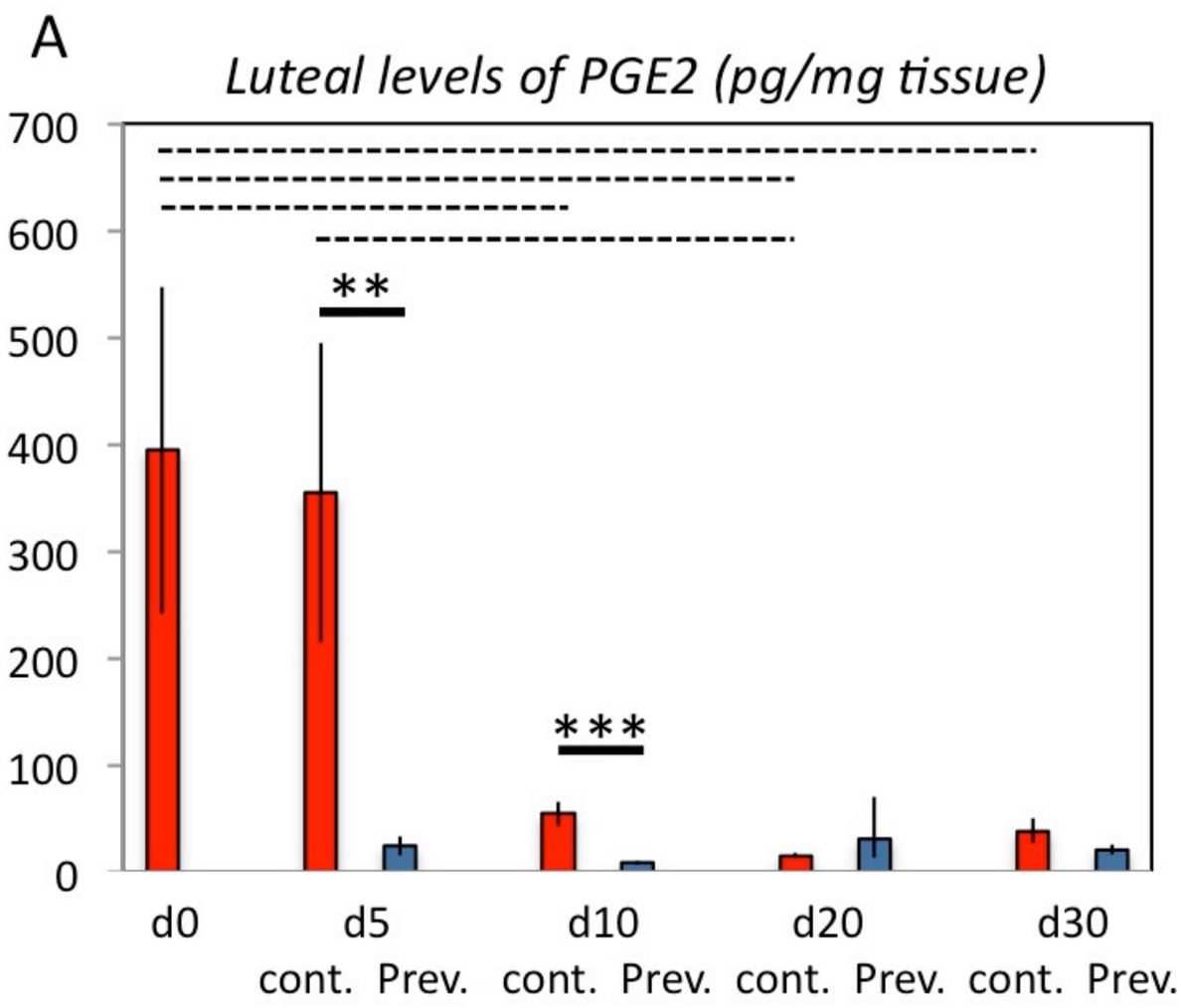


Figure 5


A
Luteal levels of PGE2 (pg/mg tissue)
700
600
500
400
300
200
100
0
**
***
d0
d5
d10
d20
d30
cont. Prev. cont. Prev. cont. Prev. cont. Prev.

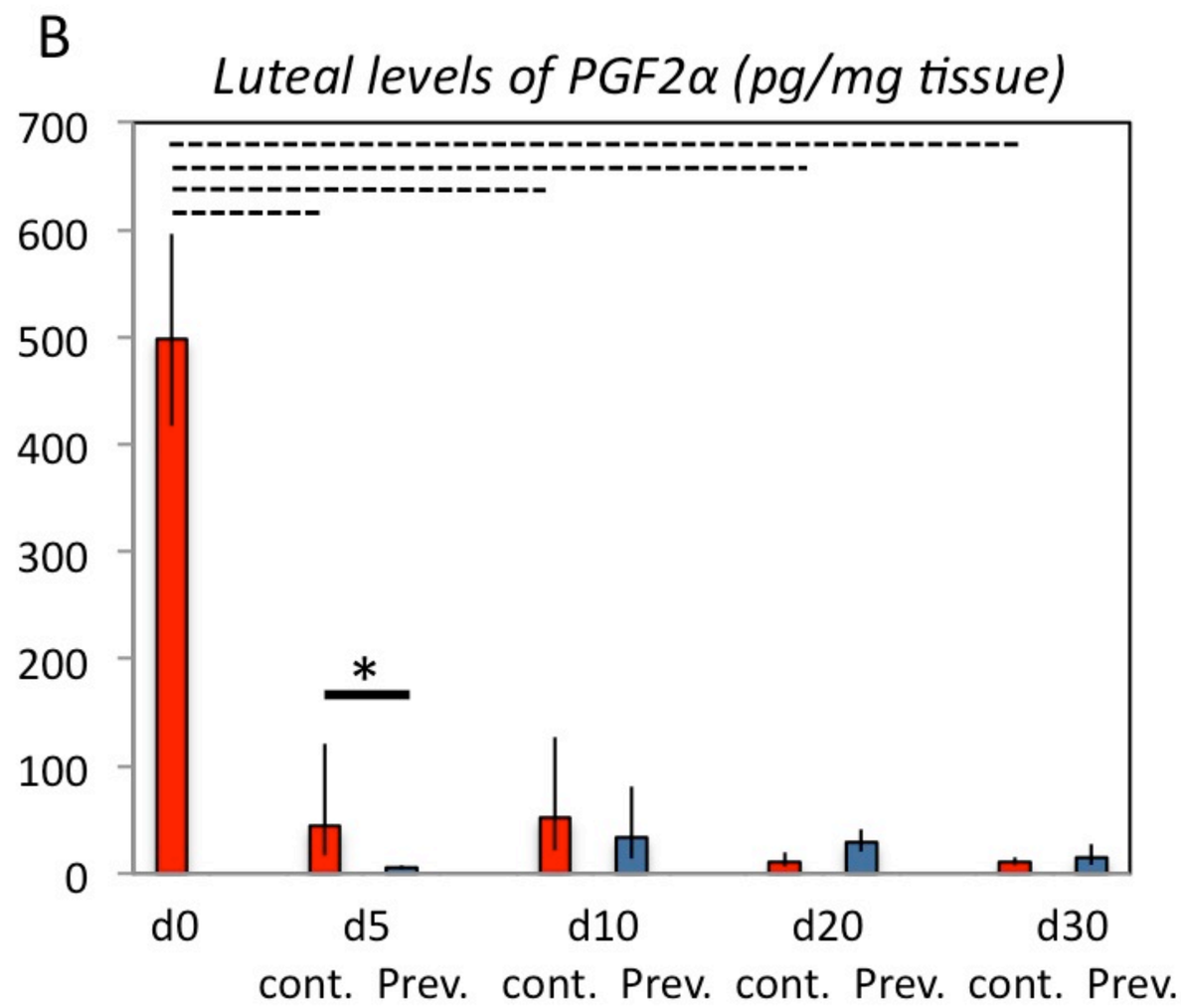

B
Luteal levels of PGF2α (pg/mg tissue)
700
600
500
400
300
200
100
0
*
d0
d5
d10
d20
d30
cont. Prev. cont. Prev. cont. Prev. cont. Prev.

Figure 6

Primer	Accession numbers	Primer Sequence	Product lenght (bp)
GAPDH (forward) GAPDH (reverse) GAPDH (TaqMan Probe)	AB028142	5'-GCT GCCAAATAT GAC GAC ATC A-3' 5'-GTA GCC CAG GAT GCC TTT GAG-3' 5'-TCC CTC CGA TGC CTG CTT CAC TAC CTT-3'	75bp
18SrRNA (forward) 18SrRNA (reverse) 18SrRNA (TaqMan Probe)	FJ797658	5'-GTC GCT CGC TCC TCT CCT ACT-3' 5'-GGC TGA CCG GGT TGG TTT-3' 5'-ACA TGC CGA CGG GCG CTG AC-3'	125bp
STAR (forward) STAR (reverse) STAR (TaqMan Probe)	EF522840	5'- CGA GGC TCC ACC TGT GTG T-3' 5'- CCT TTC TGC TCA GGC ATC TC-3', 5'- CTG GCA TGG CCA CAC ATT TC-3	65bp
PGES (forward) PGES (reverse) PGES (TaqMan Probe)	NM_001122854	5'-GTC CTG GCG CTG GTG AGT -3' 5'- ATG ACA GCC ACC ACG TAC ATC T-3' 5'-TCC CAG CCT TCC TGC TCT GCA GC -3'	89bp
PTGER2 (EP2) (forward) PTGER2 (EP2) (reverse) PTGER2 (EP2) (TaqMan Probe)	AF075602	5'- CAC CCT GCT GCT GCT TCT C-3' 5'- CGG TGC ATG CGG ATG AG-3' 5'-TGC TCG CCT GCA ACT TTC AGC GTC -3'	78bp
PTGER4 (EP4) (forward) PTGER4 (EP4) (reverse) PTGER4 (EP4) (TaqMan Probe)	NM_001003054	5'- AAA TCA GCA AAA ACC CAG ACT TG-3' 5'-GCA CGG TCT TCC GCA GAA -3' 5'-ATC CGA ATT GCT GCT GTG AAC CCT ATC C -3'	96bp
COX2 (forward) COX2 (reverse) COX2 (TaqMan Probe)	HQ110882	5'- GGA GCA TAA CAG AGT GTG TGA TGT G-3' 5'- AAG TAT TAG CCT GCT CGT CTG GAA T-3' 5'- CGC TCA TCA TCC CAT TCT GGG TGC -3'	87bp
PGT (forward) PGT (reverse) PGT (TaqMan probe)	NM_001011558	5′-TGC AGC ACT AGG AAT GCT GTT C-3′ 5′-GGG CGC AGA GAA TCA TGG A-3′ 5′-TCT GCA AAC CAT TCC CCG CGT G-3′	116bp
HPGD (forward) HPGD (reverse) HPGD (TaqMan Probe)	NM_001284477	5'-GGC AGC GAA TCT CAT GAA CAG-3' 5'- TCT TCT TTC TCA ATG GAT TCA AGGA-3' 5'- TGA ATG CCA TTT GCC CAG GCT TTG-3'	93bp
PRLr (forward) PRLr (reverse) PRLr (TaqMan Probe)	HQ267784	5'-GGA TCT TTG TGG CCG TTC TTT-3' 5'-AAG GAT GCA GGT CAC CAT GCT AT-3' 5'-ATT ATG GTC GTA GCA GTG GCT TTG AAA GGC-3'	92bp
PGR (forward) PGR (reverse) PGR (TaqMan Probe)	NM_001003074	5'-CGA GTC ATT ACC TCA GAA GAT TTG TTT -3' 5'- CTT CCA TTG CCC TTT TAA AGA AGA-3' 5'- AAG CAT CAG GCT GTC ATT ATG GTG TCC TAA CTT-3'	113bp
LHR (forward) LHR (reverse) LHR (TaqMan Probe)	XM538486	5'- TCA TCA TTT GTG CTT GCT ACA TTA AA-3' 5'- CGC CAT TTT CTT AGC AAT CTT TG-3' 5'- TGC AGT TCA AAA TCC AGA GCT GAT GGC-3'	98bp

Table 1